

False-Positive Ethanol in Clinical and Postmortem Sera by Enzymatic Assay: Elimination of Interference by Measuring Alcohol in Protein-Free Ultrafiltrate, William C. Thompson, Deepak Malhotra, David P. Schammel, Walter Blackwell, Michael E. Ward, and Amitava Dasgupta¹ (Dept. of Pathol., Office of the Med. Investigator, and Dept. of Med., Univ. of New Mexico School of Med., Albuquerque, NM; ¹ address correspondence to this author at: Pathology Services, Univ. of New Mexico Hosp., 2211 Lomas Blvd. NE, Albuquerque, NM 87106, fax 505-272-0240)

The Emit[®] alcohol assay (Syva, San Jose, CA) provides false-positive results in postmortem samples because of increased concentrations of lactate and lactate dehydrogenase (LDH) (1). Recently, we found by Emit assay falsely increased ethanol values in the sera of two living subjects with high lactate and LDH concentrations, indicating that this problem is not limited to postmortem specimens only. We investigated the possibility of eliminating interference from lactate and LDH by measuring alcohol in protein-free ultrafiltrates.

The Emit assay for ethanol was performed on a Monarch (Instrumentation Laboratory, Lexington, MA) analyzer according to the manufacturer's protocol. Alcohol concentrations were also measured by a gas-chromatographic technique with *n*-propanol as the internal standard. The gas chromatograph (Perkin-Elmer, Norwalk, CT; Model 3920) we used was equipped with a 305-cm (10-ft.) Carbowax column, which yielded baseline separation of methanol, ethanol, acetone, and isopropanol.

The protein-free ultrafiltrates of sera were prepared by centrifuging 0.8–1.0 mL of sera in a Centrifree Micropartition System (Amicon, Danvers, MA) for 20 min at 1500g. The concentrations of LDH and lactate in sera were measured with an Ektachem 700 analyzer (Eastman Kodak, Rochester, NY).

Patient 1 had end-stage renal disease secondary to type II diabetes mellitus. After receiving a cadaveric kidney transplant, she required surgical intervention for a ureteral leak. The following medications were administered: azathioprine, prednisone, cyclosporin A, nystatin, trimethoprim/sulfamethoxazole, nifedipine, alprazolam, and famotidine. About 1 week later, the patient became obtunded and developed severe metabolic acidosis with an anion gap of 20 mmol/L. The entire colon from the ileum to the sigmoid colon was found to be necrotic. Her serum concentration of LDH was 27 000 U/L and lactate 15 mmol/L before death (reference range in health, 300–600 U/L and 0.5–2.2 mmol/L, respectively). Her apparent serum ethanol concentration by Emit was 690 mg/L (Table 1).

The second patient had had an inferior wall myocardial infarction 1 week after carotid endarterectomy. The patient was not a candidate for thrombolytic therapy because of the recent surgery. LDH was 12 473 U/L and increased to 45 500 U/L the same day. The γ -glutamyltransferase, aspartate aminotransferase, alkaline phosphatase, and alanine aminotransferase activities were also increased into thousands of units per liter. The total and conjugated bilirubin concentrations were normal. The patient was hemo-

Table 1. Concentrations of ethanol measured in serum and corresponding ultrafiltrate samples.

| Sample | Ethanol, mg/L | | | Serum LDH, U/L | Serum lactate, mmol/L |
|-------------------|---------------|---------------|------------|----------------|-----------------------|
| | Emit | | Gas chrom. | | |
| | Serum | Ultrafiltrate | | | |
| Patient 1 | 690 | 0 | 0 | 27 000 | 15.0 |
| Patient 2 | 440 | 0 | 0 | 24 623 | 5.6 |
| Patient 3 | | | | | |
| Antemortem | 0 | 0 | 0 | 668 | 2.5 |
| Postmortem (2 h) | 100 | 0 | 0 | 1567 | 15.6 |
| Postmortem (24 h) | 2360 | 0 | 0 | 88 443 | 33.1 |

dynamically stabilized and renal output was increased with a renal dose of dopamine. The concentration of lactate was 5.6 mmol/L. Apparent ethanol concentration measured by Emit at the end of the first day of admission was 440 mg/L (Table 1).

A third patient initially admitted to our hospital had an antemortem ethanol concentration of 0. The apparent concentration of alcohol in postmortem sera was 100 mg/L after 2 h and increased to 2360 mg/L after 24 h. As expected, alcohol was not detected in the protein ultrafiltrate (Table 1).

In serum specimens with normal concentrations of lactate, we observed no falsely increased values in the Emit assay for alcohol. When we added 17.5 mmol/L lactate to several serum specimens (negative for alcohol) with LDH concentrations ranging from 100 to 3838 U/L, we observed no interferences in sera containing LDH <1500 U/L. In one specimen with LDH at 1755 U/L, the Emit-measured ethanol concentration after adding 17.5 mmol/L lactate was 460 mg/L.

When we supplemented with lactate aliquots of a serum specimen with an LDH concentration of 3457 U/L, the apparent alcohol concentration was undetectable at lactate concentration <4.4 mmol/L, but increased to 2500 and 3300 mg/L at 8.8 and 17.5 mmol/L of lactate, respectively. The apparent ethanol concentration (*y*) as a function of added lactate concentration (*x*) gave the following regression equation: $y = 32.2x + 3.4$ ($r = 0.95$).

Emit-measured ethanol concentrations were unaffected by ultrafiltration in five different clinical specimens that had alcohol concentrations between 2500 and 4400 mg/L (ultrafiltrate values were 97–101% of serum ethanol) and normal concentrations of LDH and lactate.

To further validate that ultrafiltrate, which contains no protein, has no matrix effect on the Emit assay of ethanol, we prepared 600 μ L of ultrafiltrate from a serum pool negative for alcohol, then supplemented both serum and ultrafiltrate with ethanol, and assayed in triplicate. The concentration of alcohol in the ultrafiltrate (mean 2050, SD 10 mg/L) was not different from serum concentration (2100, 45 mg/L). Specimens that gave false-positive alcohol concentrations by the Emit assay (no alcohol detected by gas chromatography) showed no alcohol content in protein-free ultrafiltrates of those sera reassayed by the same enzymatic technique.

In the Emit assay of alcohol, the positive interference from high LDH and lactate concentrations is caused by the catalytic conversion of lactate to pyruvate by LDH, during which also NAD^+ is converted to NADH. Measurement of the change in absorbance at 340 nm to reflect the conversion of NAD^+ to NADH is also the basis for the Emit assay of alcohol, but through the catalysis of ethanol to acetaldehyde by alcohol dehydrogenase. Removing the LDH from the sample by ultrafiltration completely eliminates the interference.

The clinical situation in which a patient has lactic acidosis may be accompanied with increased LDH released by cellular breakdown. Because clinicians will often test patients with confusion and anion gap acidosis for the presence of various alcohols (2), LDH interference in the Emit assay may lead to improper diagnoses. Therefore, we emphasize with this report that interference in the Emit assay of alcohol by high lactate and LDH concentrations does occur in clinical samples, and that these interferences can easily be eliminated by analyzing for ethanol in the protein-free ultrafiltrates.

References

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Sensitive Assay for Sodium Pump Inhibition,

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The sodium pump is essential to the function of all eukaryotic cells. The possibility that one or more pump inhibitors or digitalis-like factors (DLFs) contribute to the function of the kidney (1), the heart (2), the brain (3), the eye (4), and other systems is under active investigation. Excess or insufficient physiological amounts of these inhibitors may also contribute to disease (1-4). Attempts to isolate and characterize DLFs have often been limited by the lack of sensitivity of current $[\text{Na},\text{K}]\text{ATPase}$ assay methods and the small amount of inhibitor typically isolated. For these reasons, we undertook an effort to increase the sensitivity of the $[\text{Na},\text{K}]\text{ATPase}$ assay.

$[\text{Na},\text{K}]\text{ATPase}$ was purified from the outer medulla of calf kidney as described by Jorgensen (5), with modifications. The microsomal fraction was incubated at 25°C for 30 min with deoxycholate (1.4 g/mg protein per liter) and centrifuged through a two-layer sucrose gradient of 23% and 38% (110 min at 180 000g). The fraction between 23% and 38% of sucrose was collected, diluted, and centrifuged for 20 h at 150 000g. The pellet was resuspended in buffer (mmol/L: 250 sucrose, 30 histidine, 1 EDTA, pH 7.2) at 1 g of protein per liter and stored at 4°C. Canine renal $[\text{Na},\text{K}]\text{ATPase}$ (Sigma, St. Louis, MO) was used for comparison.

Ouabain, in graded concentrations, was used as a specific inhibitor of $[\text{Na},\text{K}]\text{ATPase}$. DLF, highly purified from the

peritoneal dialysate (PD) of renal-failure patients by ultrafiltration and two HPLC steps as described previously (1), was used for comparison. This purification separates PD-DLF completely from common steroids (cortisol, aldosterone, dehydroepiandrosterone, estrogens, progesterones, and testosterone) (1). Because PD-DLF is chemically unstable (1), all the PD-DLF from one purification was divided equally in the two assays and assayed simultaneously on the day of purification.

$[\text{Na},\text{K}]\text{ATPase}$ activity was determined by measuring the hydrolysis of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$:

Routine ATPase assay: Enzyme (10 μg) was incubated in 120 μL of buffer containing (in mmol/L) 100 Na^+ , 5 K^+ , 3 MgCl_2 , 1 EGTA, and 80 Tris (pH 7.5) for 30 min at 37°C in the presence and absence of inhibitor. The reaction was started by adding 10 μL of 40 mmol/L $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Amersham, Arlington Heights, IL; final specific activity, 70 mCi/mol) and ended after 10 min by adding 0.87 mL of 40 g/L charcoal in 0.1 mmol/L HCl solution. After centrifugation, an aliquot of the supernate was removed and its radioactivity counted. Ouabain-insensitive activity was defined as the activity remaining in the presence of 1 mmol/L ouabain. Ouabain-sensitive inhibition was calculated as the percentage reduction of counts in the presence of ouabain or DLF divided by counts in presence of buffer, after subtraction of ouabain-insensitive activity.

Modified method: The inhibitors were preincubated with $[\text{Na},\text{K}]\text{ATPase}$ in a buffer containing (in mmol/L) 10 MgCl_2 , 10 NaHPO_4 and 100 Tris (pH 7.2) at 37°C. Having determined that 85% of maximum [³H]ouabain binding occurred within 15 min, we used a preincubation of 30 min to achieve steady-state binding. After preincubation, 40 μL of preincubation mixture was transferred to 100 μL of the routine reaction buffer, equilibrated for 5 min, and 10 μL of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added. The reaction was terminated by adding 0.85 mL of the 40 g/L charcoal solution and the routine method followed thereafter. The intraassay and interassay CVs were 5.2% and 8.4%, respectively.

Plots of the ouabain concentration-dependent inhibition of $[\text{Na},\text{K}]\text{ATPase}$ hydrolysis in the routine and modified assays were parallel (Fig. 1A). The half-maximal inhibitory concentration (IC_{50}) of ouabain, measured after preincubation in 10 mmol/L Mg and 10 mmol/L phosphate was 5.3×10^{-9} mol/L, whereas the IC_{50} with preincubation in routine buffer was 1.9×10^{-7} mol/L, a 35-fold shift in sensitivity. Results were equivalent for canine renal or our purified bovine kidney $[\text{Na},\text{K}]\text{ATPase}$. Quantities of PD-DLF were insufficient to obtain full dose-response curves. PD-DLF inhibition of $[\text{Na},\text{K}]\text{ATPase}$ was significantly enhanced in the modified assay. Comparable increases were seen for ouabain solutions producing 0-15% inhibition in the routine assay, the range observed for DLF (Fig. 1B). Without the addition of ATP to begin the reaction, 50% of the enhancement was lost 20 min after transfer to the reaction solution.

Low K^+ and high Mg^{2+} and PO_4^{3-} favor ouabain binding (6), whereas Na^+ and K^+ are required for $[\text{Na},\text{K}]\text{ATPase}$ activity. We found that preincubation of ouabain or PD-DLF with enzyme under ion conditions favoring binding resulted in high-affinity binding that was not immediately reversed when the buffer was changed to the conventional assay buffer required for the hydrolysis reaction. While it was reasonable to anticipate that the buffer would enhance binding, we also thought that it would persist only as long as the low K^+ , high Mg^{2+} and